Section: Food Microbiology and Safety

Microbial activity inhibition in chilled mackerel (*Scomber scombrus*) by employment of an organic acid-icing system

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ABSTRACT

The present work concerns Atlantic mackerel (*Scomber scombrus*) traded as a chilled product. The study was aimed to investigate the effect of including a mixture of organic acids (citric, ascorbic and lactic) in the icing medium employed during the fish chilled storage. To this end and according to preliminary trials results, an aqueous solution including 0.050 % (w/v) of each acid was employed as icing medium; its effect on the microbial activity development in mackerel muscle was monitored for up to 13 days of chilled storage and compared to a counterpart-fish batch kept under traditional water ice considered as control. Results indicated a lower bacterial growth in mackerel muscle subjected to storage in the organic acid-icing system by comparison with control fish. Thus, statistically-significant ($p<0.05$) differences between both batches for all six microbial groups investigated (aerobes, anaerobes, psychrotrophes, Enterobacteriaceae, lipolytics and proteolytics) and for two chemical indices related to microbial activity development (total volatile bases and trimethylamine) were obtained. The surface wash caused by the melting of the ice during storage and the subsequent antimicrobial effect of such acids on skin microflora of the fish can be invoked as the main reasons for the limited bacterial growth found in the corresponding mackerel muscle.

Running Head: Microbial inhibition in chilled mackerel.

Keywords: *Scomber scombrus*, ascorbic, citric, lactic, chilling, microbial activity.

PRACTICAL APPLICATION

Among natural antioxidants, citric, ascorbic and lactic acids are low molecular weight organic compounds that represent a relevant choice because of their easy availability, low commercial cost and wide range of permitted concentrations for their use in foods. Present results obtained by their inclusion in a novel icing system have led to a lower microbial development in chilled mackerel when compared to its counterpart fish kept under traditional icing conditions. Such a finding indicated that inclusion of this acid mixture in the icing medium can lead to a marked quality and safety enhancement as well as to profitable commercial value increases.

INTRODUCTION

Maintain good quality and shelf life extension of fresh fish are nowadays mandatory. Flake ice has been the most employed method to cool and store fish products and partially inhibit detrimental effects on the commercial value. However, significant deterioration of sensory quality and nutritional value has been detected in chilled fish as a result of microbial and biochemical degradation mechanisms (Whittle and others 1990). To retard fish spoilage as long as possible, a wide number of preservative strategies to be combined to flake ice chilling have been tested satisfactorily such as chemical (washing or dipping by means of an aqueous solution including preservative compounds) and physical (hydrostatic high pressure, low-dose irradiation, etc.) treatments (Ashie and others 1996; Richards and others 1998) and employment of preservative packaging (Ozen and Floros 2001).

Among previous chemical treatments to chilling storage, natural low molecular weight organic acids and their sodium salts have shown to represent a relevant choice because of their easy availability, low commercial cost and wide range of permitted concentrations for their use. Thus, ascorbic and citric acids (AA and CA, respectively) are widely known for their role as chelators, acidulants in biological systems and synergists of primary antioxidants, so that a profitable effect on fish fillets (Badii and Howell 2002; Pourashouri and others 2009) and whole fish (Aubourg and others 2004) has been observed. Further, lactic acid (LA) has been reported to be effective in suppressing Gram-negative bacteria, which are known to be the most important fish spoiler group; thus, LA pre-treatment has shown to be effective in preserving and extending shelf-life in fish fillets (Kim and others 1995; Metin and others 2001), coated fish (Gogus and others 2006) and fish slices (Sallam 2007).

Small pelagic fatty fish species can constitute food products of great economic importance in many European countries (FAO 2007a). Some of these fish species are captured in high proportions when their demand is relatively low, so that a large portion of their catches is underutilized and transformed into fish meals for animals. Thus, great attention is being accorded by manufacturers in the search of appropriate technological treatments that may increase their shelf-life and accordingly, their trading value. One such abundant species at both North Atlantic coasts is Atlantic mackerel (*Scomber scombrus*) belonging to the *Scombridae* family (FAO 2007b). Although it is recognized as a healthy food, it remains underutilized because of its short chilled shelf life (up to 9-10 days). Most research has been focused on the assessement of lipid hydrolysis and oxidation as these are the most relevant mechanisms of quality loss during mackerel chilling storage (Decker and Hultin, 1990; Saeed and Howell, 2001). In this sense, different technologies have been checked to partially inhibit them (Hwang and Regenstein, 1995; Richards and others 1998). However, chilling storage of mackerel has shown an important microbial activity (Jhaveri and others 1982; Bennour and others 1991), so that great efforts should also be directed to the inhibition of this damage pathway.

The present work concerns mackerel traded as a chilled product. The study was aimed to investigate the effect of including a mixture of organic acids in the icing medium employed. To this end, an aqueous solution including CA, AA and LA was employed as icing medium; its effect on microbial activity development was monitored in mackerel muscle for up to 13 days of chilled storage.

MATERIAL AND METHODS

Icing systems

An aqueous solution containing 0.050% (w/v) of each natural organic acid (CA, AA and LA) was prepared, packed in polythene bags and kept frozen at –20ºC until use. Traditional ice was prepared starting only from water that was packed and kept frozen in the same way as the one including the organic acid mixture. Before addition to individual fishes, the different ices were ground to obtain common flakes. Organic acids encountered in the present research are regarded as safe (GRAS) for use in foods according to European and American administrations (Madrid and others 1994; Giese 1996).

Preliminary trials were carried out in order to assess a convenient concentration of the organic acid mixture used to prepare the ice. Thus, a solution combining the three organic acids in the 0.005 % to 0.250 % concentration range was preliminary evaluated. According to results obtained on the visual analysis of individual fishes, the 0.050% concentration was chosen as the most suitable for further investigation.

Raw fish, processing and sampling

Fresh mackerel (81 individuals) were caught near the Galician Atlantic coast (north-western Spain) in Autumn 2010 and transported on ice to the laboratory. The length and weight of the fish specimens were included in the following ranges: 21-25 cm and 175-230 g, respectively.

Upon arrival in the laboratory, nine individual fishes were separated and analyzed as starting raw fish (day 0); for it, three different groups (three individuals per group) were analyzed independently in order to achieve the statistical analysis (n = 3). The remaining fish were divided into two batches (36 individuals in each batch). The

first batch (preserved fish; P batch) was placed in boxes and directly surrounded by ice prepared with the organic acid-mixture above mentioned. Fish corresponding to the second batch (control batch; C batch) was placed in boxes and surrounded by traditional ice prepared with water.

In both batches, a 1:1 fish-to-ice ratio was employed. Both batches were placed in a refrigerated room (4 ºC). Boxes employed allowed draining and ice was renewed when required. Fish samples from the two different batches were taken for analysis on days 3, 6, 10 and 13. At each sampling time, nine individuals of each batch were taken for analysis, being considered into three groups (three individuals in each group) that were studied independently in order to achieve the statistical analysis (n=3).

Microbial analysis

Samples of 10 g of fish muscle were dissected aseptically from chilled fish specimens, mixed with 90 ml of 0.1% peptone water (Merck, Darmstadt, Germany), and homogenized in sterilized stomacher bags (AES, Combourg, France) as previously described (Ben-Gigirey and others 1998; Ben-Gigirey and others 1999). In all cases, serial dilutions from the microbial extracts were prepared in 0.1% peptone water.

Total aerobes were investigated by surface inoculation on plate count agar (PCA, Oxoid Ltd., London, UK), after incubation at 30ºC for 48 h. Psychrotrophes were also investigated in PCA but incubation was carried out at 7-8 ºC for 7 days. Enterobacteriaceae were investigated by pour plating using Violet Red Bile Agar (VRBA) (Merck, Darmstadt, Germany) after incubation at 37 ºC for 24 h. Microorganisms exhibiting a proteolytic or lipolytic phenotype were investigated in casein-agar medium or tributyrin-agar, respectively, after incubation at 30 ºC for 48 h, as previously described by Ben-Gigirey and others (2000).

In all cases, bacterial counts were transformed into log CFU/ g muscle before undergoing statistical analysis. All analyses were done by triplicate.

Chemical analysis of microbial activity

Total volatile base-nitrogen (TVB-N) values were measured as previously reported (Aubourg and others 1997). Briefly, fish muscle (10 g) was extracted with 6% perchloric acid (30 ml) and brought up to 50 ml. An aliquot of the acid extracts was rendered alkaline to pH 13 with 20% NaOH and then steam-distillated. Finally, the TVB-N content was determined by titration of the distillate with 10 mM HCl. Results were expressed as mg TVB-N/ 100 g muscle.

Trimethylamine-nitrogen (TMA-N) values were determined by the picrate method, as previously described by Tozawa and others (1971). This involves the preparation of a 5% trichloroacetic acid extract of fish muscle (10 g / 25 ml). Results were expressed as mg TMA-N/ 100 g muscle.

Statistical analysis

Data obtained from the different microbial and chemical analyses were subjected to the ANOVA method ($p<0.05$) to explore differences by two different ways: icing conditions effect and chilling time effect. For it, the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA) was employed. The comparison of means was performed using the least-squares difference (LSD) method. Correlation analysis among parameters (chilling time, microbial indices and chemical values) was carried out by means of the Pearson correlation coefficient (r value).

RESULTS AND DISCUSSION

Microbial count analysis

The development of aerobic bacteria was significantly ($p<0.05$) slowed down in the batch submitted to the organic acid-icing system, as compared to the control batch (Table 1). Thus, the differences in the microbial numbers reached its maximum, more than two log units, at day 6 of storage, while the average difference for aerobic mesophiles throughout storage was 1.18 log units. Remarkably, the control batch stored in traditional ice for 13 days reached levels above 10^7 CFU/ g. Bennour and others (1991) and Gram and Huss (1996) reported aerobe count to be the breakpoint for relevant microbial spoilage (Gram and Huss 1996). On contrast, the aerobe counts in the batch preserved with organic acids were round 10^6 CFU/ g at that time (Table 1). Aerobe counts as high as 10^7 CFU/ g have been reported in the skin of horse mackerel stored for 7 days at 4ºC (Kuda and others 1996). This result is quite in agreement with our results and that underscores the microbial inhibition exerted by the organic acid-icing system. In the present research, both fish batches showed fair correlation values between aerobe counts and chilling time ($r = 0.93$ and 0.84 for C- and P-fish, respectively; Table 2).

With respect to the development of anaerobic bacteria, significant ($p<0.05$) advantages derived from the use of the organic acid-icing system were also observed (Table 1). Thus, the average differences between batches C and P in the anaerobic counts throughout storage were higher than 2 log units, which means that the slowing down of anaerobic bacterial growth was over 99%. Furthermore, final counts after 13 days of storage differed in more than 3.60 log units, which clearly indicates a remarkably better control of anaerobes growth as a result of the storage in the organic

209 acid-icing system. Correlation value between aerobe counts and chilling time was only
210 good in the case of the C batch (r = 0.93; Table 2).

211 The comparative analysis of psychrotrophe counts indicated significant ($p<0.05$)
212 differences among batches after 10 days of storage (Table 1). The development of this
213 microbial group was quite limited in both batches on early storage periods, with
214 microbial numbers below 4.85 log CFU/ g units until day six, although the organic acid
215 icing system provided a remarkable protective effect. Differences between batches
216 increased on day 10, with the psychrotrophes higher than 7 log units on the control
217 batch while the organic acid icing system exhibited values below 6 log units. Even a
218 more significant effect was observed on day 13, although both batches were above the 6
219 log CFU/ g units breakpoint of microbial spoilage. The average differences between
220 batches during the whole storage period rose to 1.17 log units, this indicating a
221 remarkable slowing down of the growth of this bacterial group in the organic acid-icing
222 system. Moreover, as storage progressed higher differences in the psychrotrophe counts
223 were achieved, these reaching its maximum at day 13; at this time (day 13), a difference
224 of 1.50 log units between batches was observed. As in the case of the aerobes, the
225 psychrotrophes did not reach 10^7 CFU/ g in the muscle of the fish specimens stored in
226 the organic acid-icing system, while this value was clearly surpassed in the control
227 batch. Both fish batches showed good correlation values between psychrotrophe counts
228 and chilling time (r = 0.94 and 0.93 for C- and P-fish, respectively; Table 2), according
229 to previous research on chilled mackerel (Bennour and others 1991).

230 With respect to Enterobacteriaceae, the counts were below 1.5 log units for both
231 batches until day 10 (Table 3). These results confirm the very good initial quality of the
232 mackerel specimens employed and the limited growth of enteric bacteria during chilled
233 storage, regardless of the ice system considered. Low numbers of Enterobacteriaceae

had also been reported before by other authors for related fish species such as horse mackerel (Rodríguez and others 2005) or jack mackerel (Figueroa and others 1990), subjected to storage on flake ice and slurry ice conditions. However, in our study, the counts for this bacterial group were similar in the organic acid-icing system than in the control batch except for advanced storage times (day 13), where a difference of 1.95 log units between batches was determined. However, the fact that the counts for this bacterial group were quite low up to day 10 (below 1.30 log CFU/ g) and as a result of the ample standard deviation values obtained, no conclusion could be depicted related to any beneficial effect of the organic acid system up to day 10. The results also indicate a good control of this microbial group up to day 10 in both batches, this being probably related with the good hygienic practices and the rapid chilling of fish. However, on day 10 the microbial quality of the fish was at its limit, this leading to a rapid increase in the numbers of Enterobacteriaceae at day 13. According to this Enterobacteriaceae count distribution, fair correlation values with chilling time were only obtained for fish corresponding to the P batch ($r = 0.87$; Table 2).

This work was also aimed at evaluating the effect of the organic acid-mixture on microbial groups exhibiting specific spoilage phenotypes. Thus, the development of bacteria able to produce extracellular lipolytic enzymes has been described to negatively affect the preservation of medium-fat fish species (Rodríguez and others 2005). Likewise, the inhibition of proteolytic bacteria, able to synthesize and secrete proteolytic enzymes, has also been linked to a better preservation of fish species, due to the limitation of the physical damage of the muscle structure also reducing the formation of alkaline compounds such as ammonia and related metabolites (Venugopal 1990; Odagami and others 1994; Makarios-Laham and Lee 1993; Rodríguez and others 2003). Accordingly, the inhibition of bacteria exhibiting lipolytic and/or proteolytic

phenotypes may limit the negative effects of lipases and proteases on the quality of fish species, since these enzymes have always been characterized for retaining activity during long storage periods even under refrigeration temperatures (Alford and Pierce 1961).

With respect to the lipolytic bacteria, and as in the cases of other microbial groups studied before, significant ($p<0.05$) differences were determined between batches (Table 3). Thus, the average difference during storage was 1.41 log units, and reached its maximum at advanced stages of storage. Remarkably, differences of 1.25 and 2.73 log units were determined at 10 and 13 days of storage, respectively. According to these results, the growth of bacteria potentially involved in the lipolytic breakdown of mackerel was slowed down as a consequence of storage in the organic acid-icing system. Similar inhibition of lipolytic microorganisms was observed with other preservation methods such as ice slurries for other medium-fat and fat fish species like horse mackerel (Rodríguez and others 2005) and sardine (Campos and others 2005), respectively. Good correlation values between lipolytic counts and chilling time were only obtained for fish corresponding to the C batch ($r = 0.93$; Table 2).

With respect to the proteolytic bacteria, a similar type of behavior was observed than in the case of lipolytic ones (Table 3). Thus, the counts of proteolytic bacteria in mackerel muscle stored in the organic acid-icing system did not reach levels of 10^4 CFU/ g at any storage time, while the control batch exhibited values higher than 10^5 CFU/ g after 10 days of storage. The average difference in the counts between batches throughout storage was 1.19 log units, and the growth inhibition was more intense as storage progressed, a result that is in agreement with those observed for the majority of the microbial groups studied before. As with lipolytic counts, proteolytic ones only

283 provided fair correlation values with chilling time in the case of the C batch (r = 0.89;
284 Table 2).

285 A marked inhibitory effect of the novel icing system on several microbial groups
286 investigated was observed, this being especially remarkable in the cases of the aerobes,
287 anaerobes and lipolytic bacteria. The surface wash caused by the melting of the ice
288 during storage and the subsequent antimicrobial effect of the organic acids on skin
289 microflora of the fish can be invoked as the main reasons for the limited bacterial
290 growth found in the corresponding mackerel muscle. Similar results had been reported
291 before for other preservation methods such as ice slurries, where ice melting led to the
292 release of salt that exerted a bacteriostatic effect on fish microflora (Rodríguez and
293 others 2003; Rodríguez and others 2005; Campos and others 2005). Moreover, previous
294 studies on horse mackerel have confirmed the presence of *Proteus penneri* and
295 *Staphylococcus xylosus* strains exhibiting remarkable lipolytic and proteolytic
296 phenotype, and, to a lesser extent, *Proteus vulgaris*. The fact that the organic acid-icing
297 system evaluated in this study significantly ($p<0.05$) slowed down the growth of
298 proteolytic and lipolytic bacteria in mackerel muscle, stresses the benefits that such
299 storage system may have on the maintenance of the quality of this fish species.
300

301 **Chemical analysis of microbial activity**

302 Microbial activity development was also measured by chemical indices. The
303 TVB-N content showed a relatively high value (30.21±1.48) for starting fish (Table 4);
304 this value is found quite similar to the one obtained by Civera and others (1993) (31.9
305 mg/ 100 g muscle) but markedly higher than the values reported by Bennour and others
306 (1991) and Fagan and others (2003). In this study (Table 4), a higher ($p<0.05$) TVB-N
307 content in fish samples corresponding to the control batch could be observed when

compared to preserved fish in the 6-13-day period. Volatile amine content showed a marked increase in the 10-13-day period for C-fish, this being in agreement with a previous report on chilled mackerel (Bennour and others 1991); in the case of the P-batch, a marked increase on volatile amine formation was only attained at the end of the experiment. As for aerobe count assessment, TVB-N values showed a mean value decrease in the P-fish batch in the 0-6-day period; this result can be explained as a result of the preservative effect of the organic acid presence in the ice and by a relatively low microbial activity at that period. Good correlation values between TVB-N content and chilling time were obtained for fish corresponding to the C batch ($r = 0.91$; Table 2), according to the strong relationship between TVB-N content and freshness loss (Whittle and others 1990; Bennour and others 1991). Volatile amine compounds have been reported to be produced partially by means of endogenous enzyme activity, but mostly as a result of microbial development (Whittle and others 1990).

Initial fish showed low TMA-N values (0.07 ± 0.04; Table 4), this indicating the high freshness of the raw material employed. All fish specimens showed a progressive TMA-N content increase ($p<0.05$) with time regardless the icing system employed; such increase was specially marked at the end of storage, this also being in agreement with previous reports on chilled mackerel (Jhaveri and others 1982; Bennour and others 1991). As a result of this, good correlation values between TMA-N values and chilling time were observed ($r = 0.93$ and 0.95, for C- and P-fish, respectively; Table 2). Such results are in agreement with previous research where TMA-N assessment showed to be an accurate index for assessing quality loss during mackerel chilling storage (Bennour and others 1991; Civera and others 1993), according to the strong relationship between TMA-N content and freshness loss (Whittle and other 1990). As for TVB-N value, trimethylamine has been reported to be produced mostly as a result of microbial

development. Comparison between both kinds of icing conditions (Table 4) showed lower TMA-N mean values throughout the whole experiment as a result of employing the organic acid-mixture in the icing medium; such differences were found significant at the end of the experiment.

Relationship between chemical and microbial parameters was also analyzed in the present research (Table 5). Related to the control batch, better correlation values with microbial count assessments were obtained for TMA-N values ($r = 0.84$-0.92) than for TVB-N scores ($r = 0.78$-0.89). In this sense, previous research (Bennour and others 1991) showed that TMA-N content was profitable in order to classify the freshness degree of chilled mackerel (first, second and third grade would correspond to 0-1, 1-3 and 3-6 mg TMA-N/ 100 g muscle, respectively). In addition, a previous correlation study between TVB-N and TMA-N values and sensory acceptance in chilled mackerel concluded that this species was not suitable for consumption when both parameters were above 40 and 4 mg/ 100 g muscle, respectively (Civera and others 1993). In the present research, a higher TVB-N value was attained in the case of the control fish at the end of the experiment (Table 4).

Related to the preserved batch, correlation values were again better for TMA-N value than for TVB-N scores. For both chemical parameters, fair correlation values were obtained with aerobes, psychrotrophes and Enterobacteriaceae.

CONCLUSIONS

The results obtained in this study indicated significant ($p<0.05$) differences between batches for all six microbial groups investigated, as well as in both chemical indices related to microbial activity development. These findings clearly indicated a significantly ($p<0.05$) lower bacterial growth in mackerel muscle subjected to storage in

the organic acid-icing system as compared with traditional flake ice. The novel icing system evaluated in this work contains three organic acids, all of them previously reported as possessing antimicrobial and antioxidant activities. In addition, such natural organic acids are known to represent a relevant choice because of their easy availability, low commercial cost and wide range of permitted concentration for their use in foods.

As being a highly fatty fish species, previous research on quality loss of chilled mackerel has mostly focused the lipid changes, these concerning specially lipid oxidation and hydrolysis. In the present investigation, an extensive study on microbial activity development during the chilled storage of this pelagic fish species has been undertaken; as a result, progress of different bacteria groups has been described, as well as its relationship with chemical parameters related to microbial activity development.

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TABLE 1

Aerobe, anaerobe and psychrotrophe count (log CFU/ g muscle) assessment* in chilled mackerel kept under different icing conditions**

Chilling Time (days)	Aerobes		Anaerobes		Psychrotrophes	
	C	P	C	P	C	P
Raw fish	3.31 C (0.08)	3.31 B (0.08)	1.97 D (0.85)	1.97 AB (0.85)	3.26 C (0.37)	3.26 B (0.37)
3	3.41 BC (0.65)	3.12 B (0.19)	3.33 C (0.60)	2.79 A (0.70)	4.02 BC (0.61)	3.16 B (1.02)
6	4.21 aB (0.37)	2.20 bC (0.17)	3.50 aC (0.41)	1.99 bB (0.05)	4.85 B (0.30)	3.77 B (1.06)
10	6.44 aA (0.45)	5.34 bA (0.52)	4.57 aB (0.43)	1.99 bB (0.05)	7.06 aA (0.37)	5.74 bA (0.45)
13	7.28 aA (0.32)	6.05 bA (0.77)	5.68 aA (0.44)	1.99 bB (0.05)	7.93 aA (0.50)	6.43 bA (0.51)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different low-case letters (a, b) indicate significant ($p<0.05$) differences as a result of the icing condition. For each parameter and for each icing condition, values followed by different capital letters (A-D) denote significant ($p<0.05$) differences as a result of the chilling time. No letters are indicated when significant differences are not found ($p>0.05$).

** Abbreviations of icing conditions: P (ice including the organic acid-mixture) and C (ice prepared only from water).

TABLE 2

Correlation coefficient (r value)* between the chilled storage time and the different parameters analyzed in chilled mackerel kept under different icing conditions**

Quality parameter	r value	
	C	P
Aerobes	0.93 [b]	0.84 [b]
Anaerobes	0.93 [a]	-0.39 [b]
Psychrotrophes	0.94 [a]	0.93 [b]
Enterobacteriaceae	0.79 [b]	0.87 [b]
Lipolytics	0.93 [b]	0.80 [b]
Proteolytics	0.89 [a]	0.69 [c]
Total volatile base-nitrogen	0.91 [b]	0.79 [b]
Trimethylamine-nitrogen	0.93 [b]	0.95 [b]

* For each index, linear[a], quadratic[b] and logarithmic[c] fittings were studied. In each case, the best correlation coefficient value is expressed.

** Abbreviations of icing conditions as expressed in Table 1.

TABLE 3

Enterobacteriaeceae, lipolytic and proteolytic count (log CFU/ g muscle) assessment* in chilled mackerel kept under different icing conditions**

Chilling Time (days)	Enterobacteriaceae		Lipolytics		Proteolytics	
	C	P	C	P	C	P
Raw fish	0.99 B (0.01)	0.99 B (0.01)	2.16 D (0.28)	2.16 AB (0.28)	2.09 C (0.18)	2.09 B (0.18)
3	0.99 B (0.05)	0.99 B (0.05)	3.32 C (0.56)	2.85 A (0.41)	3.51 B (0.11)	3.16 AB (1.01)
6	0.99 B (0.05)	1.23 AB (0.41)	3.30 aC (0.52)	2.10 bB (0.18)	3.10 B (0.35)	2.76 AB (0.69)
10	0.99 B (0.05)	1.29 AB (0.53)	4.81 aB (0.07)	3.56 bAB (1.15)	5.30 aA (0.29)	3.78 bA (1.09)
13	4.42 aA (0.44)	2.47 bA (0.93)	6.40 aA (0.20)	3.67 bAB (1.85)	5.51 aA (0.52)	2.96 bAB (1.48)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different low-case letters (a, b) indicate significant ($p<0.05$) differences as a result of the icing condition. For each parameter and for each icing condition, values followed by different capital letters (A-D) denote significant ($p<0.05$) differences as a result of the chilling time. No letters are indicated when significant differences are not found ($p>0.05$).

** Abbreviations of icing conditions as expressed in Table 1.

TABLE 4

Evolution of total volatile base-nitrogen (TVB-N) and trimethylamine-nitrogen (TMA-N) values* in chilled mackerel kept under different icing conditions**

Chilling Time (days)	TVB-N (mg/ 100 g muscle)		TMA-N (mg/ 100 g muscle)	
	C	P	C	P
Raw fish	30.21 C (1.48)	30.21 B (1.48)	0.07 D (0.04)	0.07 D (0.04)
3	30.74 C (2.00)	28.69 BC (1.70)	0.29 CD (0.20)	0.16 D (0.04)
6	30.15 aC (1.57)	27.44 bC (0.62)	0.54 C (0.27)	0.34 C (0.06)
10	35.12 aB (2.08)	30.47 bB (1.57)	1.04 B (0.12)	0.85 B (0.14)
13	44.61 aA (1.76)	35.21 bA (2.53)	2.32 aA (0.25)	1.47 bA (0.23)

* Mean values of three replicates (n = 3); standard deviations are indicated in brackets. For each parameter and for each chilling time, mean values followed by different low-case letters indicate significant ($p<0.05$) differences as a result of the icing condition. For each parameter and for each icing condition, values followed by different capital letters (A-D) denote significant ($p<0.05$) differences as a result of the chilling time. No letters are indicated when significant differences are not found ($p>0.05$).

** Abbreviations of icing conditions as expressed in Table 1.

TABLE 5

Linear correlation coefficient values between microbial and chemical parameters analyzed in chilled mackerel kept under different icing conditions*

Microbial parameter	Total volatile base-nitrogen		Trimethylamine-nitrogen	
	C	P	C	P
Aerobes	0.86	0.84	0.88	0.84
Anaerobes	0.84	-0.30	0.84	-0.37
Psychrotrophes	0.84	0.75	0.88	0.92
Enterobacteriaceae	0.89	0.84	0.87	0.88
Lipolytics	0.89	0.68	0.92	0.80
Proteolytics	0.78	0.07	0.88	0.42

* Abbreviations of icing conditions as expressed in Table 1.